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Nonhalogenated Alkane Anesthetics Fail to Potentiate Agonist Actions on Two Ligand-gated Ion Channels

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Background: **Although ether, alcohol, and halogenated alkane anesthetics potentiate agonist actions or increase the apparent agonist affinity of ligand-gated ion channels at clinically relevant concentrations, the effects of nonhalogenated alkane anesthetics on ligand-gated ion channels have not been studied. The current study assessed the abilities of two representative nonhalogenated alkane anesthetics (cyclopropane and butane) to potentiate agonist actions or increase the apparent agonist affinity of two representative ligand-gated ion channels: the nicotinic acetylcholine receptor and γ-aminobutyric acid type A ($GABA_A$) receptor.**

Methods: **Nicotinic acetylcholine receptors were obtained from the electroplax organ of *Torpedo nobiliana*, and human $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2L}$) were expressed in human embryonic kidney 293 cells. The *Torpedo* nicotinic acetylcholine receptors apparent agonist affinity in the presence and absence of anesthetic was assessed by measuring the apparent rates of desensitization induced by a range of acetylcholine concentrations. The $GABA_A$ receptor's apparent agonist affinity in the presence and absence of anesthetic was assessed by measuring the peak currents induced by a range of GABA concentrations.**

Results: **Neither cyclopropane nor butane potentiated agonist actions or increased the apparent agonist affinity (reduced the apparent agonist dissociation constant) of the *Torpedo* nicotinic acetylcholine receptor or $GABA_A$ receptor. At clinically relevant concentrations, cyclopropane and butane reduced the apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization induced by low concentrations of agonist.**

Conclusions: **Our results suggest that the *in vivo* central nervous system depressant effects of nonhalogenated alkane anesthetics do not result from their abilities to potentiate agonist actions on ligand-gated ion channels. Other targets or mechanisms more likely account for the anesthetic activities of nonhalogenated alkane anesthetics.**

THE *Torpedo* nicotinic acetylcholine receptor (nAchR), γ-aminobutyric acid type A receptor ($GABA_AR$), the glycine receptor, and 5-hydroxytryptamine$_3$ receptor together form an anesthetic-sensitive superfamily of ligand-gated ion channels (LGICs) that are critical for synaptic function.[1,2] Members of this superfamily are widely considered to be the most important targets of general anesthetics and are frequently used in studies of anesthetic molecular mechanisms.[3–11] Such studies have shown that, at clinically relevant concentrations (≤ 3 minimum alveolar concentration [MAC]), general anesthetics representing a wide range of chemical classes (including halogenated alkanes, halogenated and nonhalogenated ethers, and alcohols) increase ion flux or apparent desensitization rates induced by low agonist concentrations. When experiments have been performed using a range of agonist concentrations, this action has been shown to reflect a reduction in the receptor's apparent agonist dissociation constant (K_d) (an increase in apparent agonist affinity).[3,8,12–15]

Despite a large body of literature documenting the effects of anesthetics on a variety of LGICs, there have been no studies to assess the effects of nonhalogenated alkane anesthetics on any LGIC. Nonhalogenated alkanes, in particular cyclopropane, were commonly used in the past as clinical inhalational anesthetics before they were supplanted by nonflammable halogenated analogs. The goal of the current study was to test the hypothesis that agonist potentiation of LGICs might also play an important role in the clinical actions of nonhalogenated alkane anesthetics. This was achieved by assessing the abilities of cyclopropane and butane to increase the apparent agonist affinities of the two best characterized LGICs: the nAchR and the $GABA_AR$. Our results reveal that, in contrast to nearly all other previously studied anesthetics, cyclopropane and butane do not potentiate agonist actions on LGICs at clinically relevant concentrations. This suggests that other molecular mechanisms account for the *in vivo* anesthetic activities of nonhalogenated alkane anesthetics.

Materials and Methods

Torpedo nobiliana was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate and acetylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). Alkane anesthetics were purchased from Aldrich (Milwaukee, WI). The fluorescent partial agonist, [1-(5-dimethylaminonaphthalene)sulfonamido] n-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester (Dns-C_6-Cho), was synthesized according to the procedure of Waksman *et al.*[16]

Preparation and Characterization of Nicotinic Acetylcholine Receptor–rich Membranes

Receptor membranes were obtained from freshly dissected *T. nobiliana* electric organs and prepared using sucrose density gradient centrifugation as described by Braswell *et al.*[17] and in accordance with guidelines es-

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tablished by the Massachusetts General Hospital Animal Care Committee (Boston, MA). Membranes were stored at −80°C in *Torpedo* physiologic solution (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.02% NaN_3, pH 7.0) and thawed on the day of use. Acetylcholinesterase activity was inhibited by exposing membranes to 1.0 mM diisopropylfluorophosphate for 60 min before dilution with *Torpedo* physiologic solution to the desired receptor concentration. The number of agonist binding sites was determined from Dns-C_6-Cho titrations as previously described.[18]

Determination of the Apparent Rate of Acetylcholine-induced Desensitization

The apparent rate of agonist-induced desensitization was determined with a double-agonist pulse assay using a sequential mixing stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, United Kingdom).[8] In brief, receptor-rich membranes were first preincubated with acetylcholine for periods ranging from 15 ms to several minutes. The number of receptors able to be activated (nondesensitized) that remained after this preincubation period was then quantified from the amplitude of the rapid fluorescence signal observed when the membrane–acetylcholine solution was rapidly mixed with an assay solution containing the fluorescent partial agonist Dns-C_6-Cho and a high, channel-activating concentration of acetylcholine (5 mM). In each experiment, receptor membranes (0.8-μM agonist binding sites) were loaded into one of the spectrofluorometer's gas-tight premix syringes, and acetylcholine was loaded into the other gas-tight premix syringe. The solutions were rapidly mixed (1 ms mixing time; 1:1 vol:vol) and allowed to preincubate for the desired time. The nAchR–acetylcholine solution was then mixed (1 ms mixing time; 1:1 vol:vol) with an assay solution containing 10 mM acetylcholine and 20 μM Dns-C_6-Cho, and the fluorescence emission was recorded. Where appropriate, solutions also contained anesthetic at the desired concentration. Solutions remained within a closed system made of inert materials (glass and Teflon), and anesthetic loss during experiments was negligible as determined by gas chromatographic analysis. An excitation wavelength of 290 nm was provided by a 150-W xenon arc lamp, and the monochromator bandpass was 5 nm. Fluorescence emission greater than 500 nm was measured through a high-pass filter. Fluorescence intensity was recorded for 500 ms after the second mixing step. All experiments were performed at 20 ± 0.3°C.

Cell Culture and Transfection with Human γ-Aminobutyric Acid Type A Receptor cDNAs

Human embryonic kidney cells (HEK 293; American Type Culture Collection, Rockville, MD) were cultured in minimum essential medium Eagle (ATCC), supplemented with 10% horse serum, 1% penicillin–streptomycin (all from Gibco BRL, Life Technologies, Grand Island, NY), and maintained at 37°C in a 5% CO_2 incubator. For transient transfection, the calcium phosphate precipitation technique was used as described by Chen and Okayama.[19] Cells were plated on protamine-coated glass coverslips and transfected with cDNAs for the α_1, β_2, and γ_{2L} subunits of human $GABA_AR$ in pCDM8 vectors with a wt/wt ratio of 1α:2β:5γ. Cells were cotransfected with an expression plasmid (πH3-CD8) for the lymphocyte surface antigen CD8-α, which was supplied by Dr. Gary Yellen (Harvard Medical School, Boston, MA). After transfection, cells were incubated for 36–72 h. Shortly before experiments, cells were incubated briefly with polystyrene microspheres, precoated with anti-CD8 antibody (Dynabeads M-450 CD8, Dynal, Great Neck, NY). Thus, cells expressing CD8 were decorated with beads and could be distinguished from untransfected ones.[20]

γ-Aminobutyric Acid Type A Receptor Electrophysiology

For electrophysiology, the medium was replaced by an extracellular solution with 162 mM NaCl, 5.3 mM KCl, 0.67 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 15 mM HEPES, 5.6 mM glucose, and 2 mM $CaCl_2$, adjusted to a pH of 7.30 with NaOH. Patch pipettes were fire-polished, resulting in open tip resistances of 2–5 MΩ. Pipettes were filled with intracellular solution containing 140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, and 10 mM glucose, adjusted to a pH of 7.30 with KOH. GABA solutions were prepared shortly before the experiments. Recordings were performed at room temperature (20–22°C) using standard outside-out or whole cell patch clamp techniques. Patches or cells were clamped to −50 mV during recordings; series resistance was compensated. Currents through the patch clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) were filtered (eight-pole bessel, 2 kHz) and digitized at 5–10 kHz using commercial software (pClamp 8.0, Axon Instruments). Rapid superfusate switching (0.2–1.2 ms, measured with open pipette junction currents) was performed using a piezo-driven quad tube as previously described.[21] Standard protocols repetitively applied different concentrations of GABA alone or in combination with cyclopropane, butane, isoflurane, or octanol at the desired concentrations as pulses lasting 1.5–2.5 s. When anesthetics were applied, they were applied simultaneously with GABA and without preincubation. A 20-s interval between pulses was used for recovery of channel activity from desensitization. Control currents elicited with GABA alone were checked before and after application of anesthetic. Data were analyzed only if precontrol and postcontrol peak currents differed less than 10%. Cyclopropane and butane were stored in airtight syringes during the experiments. All parts of the application system consist of glass or Teflon tubing material.

Neuronal Nicotinic and N-*methyl-*D*-aspartate Receptor Studies*

Xenopus frogs were maintained and treated in accordance with regulations specified by the Massachusetts General Hospital Animal Care Committee (Boston, MA). Methods for oocyte expression of ion channels have been previously described.[22] Capped mRNAs were synthesized *in vitro* from linearized cDNA templates using SP6 or T7 RNA polymerase kits (Ambion, Austin, TX) and purified with affinity beads (BIO-101, Vista, CA). Oocytes were injected with mixtures of mRNAs (20–100 ng) encoding neuronal nAchR subunits α_4 and β_2 (wt/wt ratio = 1) or *N*-methyl-D-aspartate (NMDA) receptor subunits NR1b and NR2a (wt/wt ratio = 1) and incubated at 18°C for 48–96 h. For two-microelectrode electrophysiology, oocytes were placed in a 0.1-ml perfusion chamber at room temperature (20–22°C) and voltage clamped at −50 mV *via* glass electrodes filled with 3 M KCl (0.2–1 MΩ). Oocytes expressing neuronal nAchRs were superfused with calcium-free ND-96 (96 mM NaCl, 2 mM KCl, 1 mM $BaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, pH 7.6) at 2–3 ml/min, and currents were activated with buffer containing 200 μM acetylcholine. Oocytes expressing NMDA receptors were superfused in calcium- and magnesium-free ND-96 ($MgCl_2$ replaced with $BaCl_2$) containing 10 μM glycine, and currents were activated with 100 μM NMDA. Near the middle of a 60-s agonist pulse, anesthetic–agonist solutions (delivered from closed glass syringe reservoirs *via* Telfon tubing and valves) were introduced for 12–16 s. Currents were digitized at 50–200 Hz and recorded on a personal computer.

Preparation of Anesthetic Solutions

Saturated solutions of cyclopropane or butane were made by bubbling the gases though the desired buffer solution (25–50 ml) within a 250-ml glass bottle sealed with a Teflon septum. A gas inlet and outlet line passed into the bottle though the septum. During the 1–2-h equilibration period at room temperature, solutions were continuously stirred to achieve a saturated aqueous solution. After equilibration, the outlet line was cleared by aspiration, and the saturated solutions were drawn into a gas-tight syringe that was then connected directly to the spectrofluorometer (for nAchR studies) or application system (for $GABA_A$R, neuronal nAchR, and NMDA receptor studies) *via* a Teflon stopcock. In parallel experiments using the same technique, the concentrations of cyclopropane and butane in saturated solutions (10.6 and 1.1 mM, respectively) were determined by gas chromatography to be within 10% of that predicted by their aqueous:gas partition coefficients.[23] Saturated solutions of isoflurane (15 mM) and octanol (4.5 mM) were prepared by mixing excess anesthetic with buffer overnight. To achieve the desired anesthetic concentration, saturated solutions were diluted with buffer within a gas-tight syringe.

Conversion of Minimum Alveolar Concentration to Aqueous EC_{50}

The approach for converting MAC values into aqueous EC_{50} values (and *vice versa*) has been described by Franks and Lieb.[24] All MAC values for inhalational anesthetics are for humans.[23] For butane, MAC was approximated as the EC_{50} value reported by Firestone *et al.*[23] For ethanol and octanol, MAC values are for rats.[25]

Statistical Analysis

Each data point represents the mean of at least three measurements, and the error bars indicate the SD. Statistical analysis was performed using an unpaired Student *t* test and analysis of variance (Prism version 3.0, Graphpad Software, San Diego, CA). $P < 0.05$ was considered significant. Data points on acetylcholine concentration-apparent desensitization rate curves were fit to a Hill equation in the form:

$$k_{app} = k_{max} \cdot \left(\frac{[\text{Acetylcholine}]^n}{[\text{Acetylcholine}]^n + (K_d^{app})^n} \right) \quad (1)$$

where k_{app} is the experimentally determined apparent rate of desensitization at each acetylcholine concentration, k_{max} is the maximum apparent rate of desensitization induced by high acetylcholine concentrations, K_d^{app} is the nAchR's apparent K_d for acetylcholine, and n is the Hill coefficient. The reported errors for all fitted parameters are the SDs derived from the curve fit.

For electrophysiolgic experiments using the $GABA_A$R, an analogous Hill equation was used:

$$I = I_{max} \cdot \left(\frac{[\text{GABA}]^n}{[\text{GABA}]^n + (K_d^{app})^n} \right) \quad (2)$$

where I is the experimentally determined peak current at each GABA concentration, I_{max} is the maximal peak current induced by high GABA concentrations, K_d^{app} is the $GABA_A$R's apparent K_d for GABA, and n is the Hill coefficient. Again, the reported errors for all fitted parameters are the SDs derived from the curve fit.

Results

Anesthetic Action on the Nicotinic Acetylcholine Receptor's Apparent Agonist Affinity

In the presence or absence of anesthetic, the apparent rate of *Torpedo* nAchR desensitization increased with acetylcholine concentration before reaching a maximum at high concentrations (fig. 1A). From the acetylcholine concentration dependence of the apparent rate of acetylcholine-induced desensitization, we calculated the receptor's apparent K_d for this agonist (equation 1). In the absence of anesthetic, *Torpedo* nAchRs in this mem-

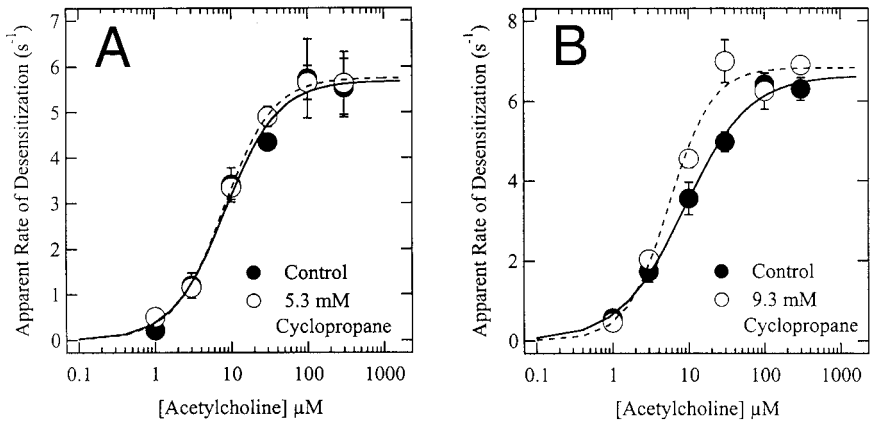

Fig. 1. The apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization as a function of acetylcholine concentration in the presence and absence of cyclopropane. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 1. (*A*) The (lack of) effect of 5.3 mM cyclopropane on the apparent rates of acetylcholine-induced desensitization. In the absence of anesthetic (control), the apparent K_d for acetylcholine in this preparation was 8 ± 2 μM, the maximum rate of desensitization was 5.7 ± 0.3 s^{-1}, and the Hill coefficient was 1.2 ± 0.2. The presence of 5.3 mM cyclopropane did not significantly change these values, as the apparent K_d was 7.9 ± 0.5 μM, the maximum rate of desensitization was 5.8 ± 0.1 s^{-1}, and the Hill coefficient was 1.3 ± 0.1. (*B*) The effect of 9.3 mM cyclopropane on the apparent rates of acetylcholine-induced desensitization. In the absence of anesthetic, the apparent K_d in this preparation was 9 ± 1 μM, the maximum rate of desensitization was 6.6 ± 0.3 s^{-1}, and the Hill coefficient was 1.0 ± 0.1. In the presence of 9.3 mM cyclopropane, the apparent K_d was 6 ± 1 μM, the maximum rate of desensitization was 6.8 ± 0.4 s^{-1}, and the Hill coefficient was 1.5 ± 0.4.

brane preparation had an apparent K_d for acetylcholine of 8 ± 2 μM and a maximum rate of desensitization of 5.7 ± 0.3 s^{-1}. Preequilibration with 5.3 mM cyclopropane (a one-half saturated aqueous solution and equivalent to 6.5 MAC) had no effect on either the apparent K_d for acetylcholine or the maximal rate of acetylcholine-induced desensitization (7.9 ± 0.5 μM and 5.8 ± 0.1 s^{-1}). Figure 1B shows the acetylcholine concentration dependence of the apparent rate of *Torpedo* nAchR desensitization in the absence of anesthetic and in the presence of 9.3 mM cyclopropane (equivalent to 11.4 MAC) using a different receptor preparation. In the absence of anesthetic, *Torpedo* nAchRs in this membrane preparation had an apparent K_d for acetylcholine of 9 ± 1 μM and a maximum rate of desensitization of 6.6 ± 0.3 s^{-1}. Preequilibration with 9.3 mM cyclopropane reduced the apparent K_d for acetylcholine by just 30% to 6 ± 1 μM (not statistically significant) and had no detectable effect on the maximal rate of desensitization.

Figure 2 shows the effect of butane on the acetylcholine concentration–response curve for *Torpedo* nAchR desensitization. At a concentration of 0.55 mM (equivalent to 3 MAC), butane failed to reduce the *Torpedo* nAchR's apparent K_d for acetylcholine (10 ± 1 μM *vs.* a control value of 8 ± 2 μM) or to alter the maximal rate of desensitization (5.6 ± 0.2 s^{-1} *vs.* a control value of 5.7 ± 0.3 s^{-1}).

To further assess the agonist-potentiating abilities of cyclopropane and butane and to compare them with other anesthetics, we measured the abilities of anesthetics to increase the apparent rate of *Torpedo* nAchR desensitization induced by 1 μM acetylcholine, an EC_{10} agonist concentration (fig. 3). We used a single-receptor preparation to more easily compare the effects of different anesthetics on the kinetics of agonist-induced desensitization without the potentially confounding effects of preparation-to-preparation variability on anesthetic sensitivity. At a concentration of 0.22 mM (0.8 MAC), isoflurane increased the apparent rate of acetylcholine-induced desensitization by 56% from a control value of 0.36 ± 0.05 s^{-1} to 0.56 ± 0.01 s^{-1}. The apparent rate of desensitization increased with isoflurane concentration, with no evidence of saturation over the concentration range studied. By 0.88 mM, the highest concentration studied, isoflurane increased the apparent desensitization rate to 1.4 ± 0.2 s^{-1}, a fourfold increase over the control. Ethanol and octanol also increased the apparent rate of acetylcholine-induced desensitization at pharmacologically relevant concentrations and in a concentration-dependent manner. However, at relevant concentra-

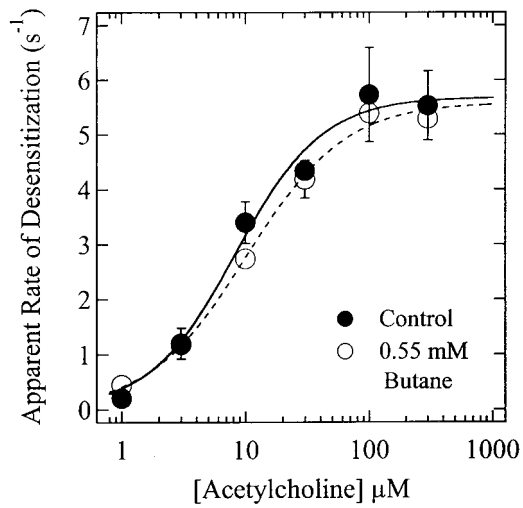

Fig. 2. The apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization as a function of acetylcholine concentration in the absence of anesthetic (control) and in the presence of 0.55 mM butane (3 MAC). Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 1. In the absence of anesthetic, the apparent K_d for acetylcholine was 8 ± 2 μM, the maximum rate of desensitization was 5.7± 0.3 s^{-1}, and the Hill coefficient was 1.2 ± 0.2. The presence of 0.55 mM butane did not significantly change these values, as the apparent K_d was 10 ± 1 μM, the maximum rate of desensitization was 5.6 ± 0.2 s^{-1}, and the Hill coefficient was 1.1 ± 0.1.

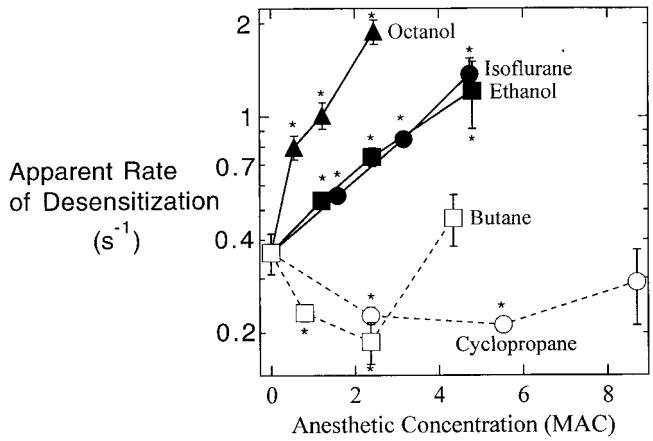

Fig. 3. Anesthetic concentration dependence of the apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization induced by 1 μM acetylcholine. At all concentrations studied, isoflurane, ethanol, and octanol significantly increased the apparent rate of desensitization. Conversely, cyclopropane and butane either reduced or had no effect on the apparent rate of desensitization. The MAC of isoflurane, butane, and cyclopropane were calculated as described in Methods as 0.28, 0.16, and 0.82 mM, respectively. The MAC values of ethanol (116 mM) and octanol (37 μM) are derived from Fang *et al.*[25] *$P < 0.05$ *versus* control (no anesthetic).

tions, cyclopropane and butane actually reduced the apparent rate of acetylcholine-induced desensitization rate. At higher, supraphysiologic (toxic) concentrations, the apparent desensitization rate was not significantly different from control.

Anesthetic Action on the γ-Aminobutyric Acid Type A Receptor's Apparent Agonist Affinity

The inset in figure 4A shows typical current traces induced by 3 μM GABA (an EC_5 agonist concentration) in the absence of anesthetic (control) and in the presence of 1.6 MAC cyclopropane. Cyclopropane did not potentiate currents induced by this low concentration of GABA. For comparison, the inset also shows the significant potentiating effect of 1.6 MAC isoflurane on currents using the same patch. The effect of 1.6 MAC cyclopropane on the $GABA_AR$'s apparent agonist K_d was determined by fitting plots of the normalized peak current as a function of GABA concentration in the presence or absence of cyclopropane to equation 2 (fig. 4A). Using this analysis, it was determined that cyclopropane did not reduce the apparent agonist K_d as the apparent K_d values for GABA in the absence and presence of cyclopropane were 23 ± 3 and 33 ± 5 μM, respectively. The inset in figure 4B shows typical current traces induced by 3 μM in the absence of anesthetic and in the presence of 1.6 MAC butane and 1.6 MAC octanol. Although octanol significantly potentiated currents induced by 3 μM GABA, butane had no effect. The plot in figure 4B shows the effect of 1.6 MAC butane on the GABA concentration–response relation. In common with cyclopropane, 1.6 MAC butane failed to significantly potentiate currents induced by this low concentration of agonist or to significantly reduce the $GABA_AR$'s apparent K_d for GABA (18 ± 2 μM as compared with a control value of 23 ± 3 μM).

Inhibition of Neuronal Nicotinic Acetylcholine Receptors and N*-methyl-*D*-Aspartate Receptors*

Because our results indicated that nonhalogenated alkane anesthetics do not potentiate LGICs, we considered the possibility that they might act, instead, by inhibiting receptor function. To test this hypothesis, we determined whether cyclopropane and butane inhibit ion flux through neuronal nAchRs and NMDA receptors. Figures 5A and B demonstrate that clinically relevant concentrations of cyclopropane inhibit ion flux through neuronal

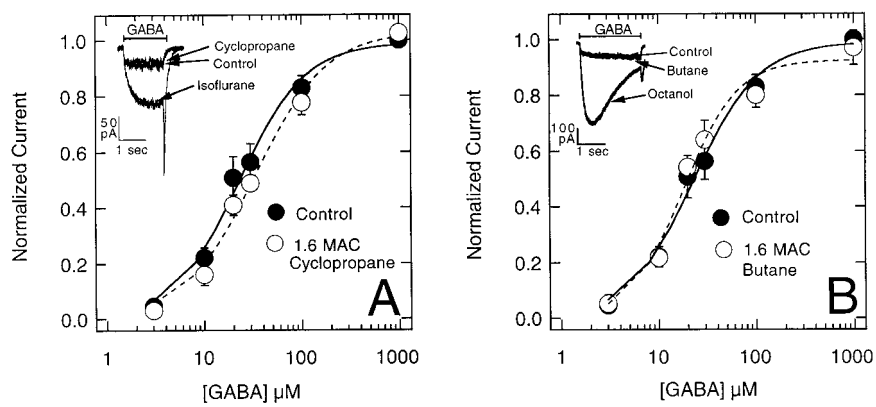

Fig. 4. The effect of 1.6 MAC cyclopropane and butane on γ-aminobutyric acid (GABA) concentration–response curves mediated by GABA type A receptors expressed in HEK 293 cells. The inset in (*A*) shows representative current traces induced by 3 μM GABA and demonstrates the lack of effect of 1.6 MAC (1.3 mM) cyclopropane. For comparison, a current trace obtained in the presence of 1.6 MAC (0.44 mM) isoflurane is also shown. (*A*) The current response to GABA in the absence and presence of cyclopropane is also plotted. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 2. Currents were normalized to that elicited by 1 mM GABA, a saturating GABA concentration. In the absence of anesthetic, the apparent K_d for GABA was 23 ± 3 μM, the maximal current was 0.99 ± 0.05, and the Hill coefficient was 1.3 ± 0.2. In the presence of cyclopropane, the apparent K_d was 33 ± 5 μM, the maximal current was 1.03 ± 0.05, and the Hill coefficient was 1.2 ± 0.2. The inset in (B) demonstrates the lack of effect of 1.6 MAC (0.26 mM) butane on the current induced by 3 μM GABA. For comparison, a current trace obtained in the presence of 1.6 MAC (58 μM) octanol is also shown. (*B*) The normalized current response to GABA application in the absence and presence of butane is also plotted. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 2. In the absence of anesthetic, the apparent K_d for GABA was 23 ± 3 μM, the maximal current was 0.99 ± 0.01, and the Hill coefficient was 1.3 ± 0.2. In the presence of 1.6 MAC butane, the apparent K_d, the maximal current was 0.92 ± 0.05, and the Hill coefficients were 18 ± 2 and 1.6 ± 0.3 μM, respectively.

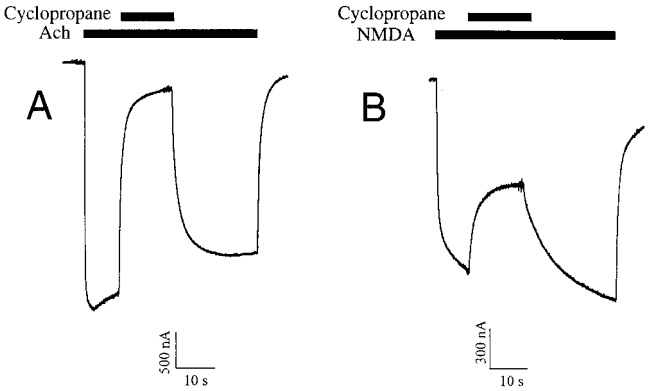

Fig. 5. The effect of cyclopropane on rat neuronal nicotinic acetylcholine receptor (nAchRs; α_4 and β_2) and human *N*-methyl-D-aspartate (NMDA) receptors (NR2A and NR1b). (*A*) The inhibition of neuronal nAchRs caused by a pulse of 1.6 MAC cyclopropane on currents induced by 100 μM acetylcholine. Oocytes were voltage clamped at −50 mV. (*B*) The inhibition of NMDA receptors caused by a pulse of 0.8 MAC cyclopropane on currents induced by 100 μM NMDA. Oocytes were voltage clamped at −100 mV, and the buffer contained only Ba^{2+} as the divalent cation along with 10 μM glycine.

nAchRs and NMDA receptors. Similarly, butane inhibits both neuronal nAchRs and NMDA receptors at clinically relevant concentrations (data not shown).

Discussion

The nAchR and $GABA_AR$ are the two best characterized members of the superfamily of LGICs. Functional studies indicate that the binding of two agonist molecules induces resting state receptors to undergo a rapid conformational transition to an ion-permeable open-channel state.[26–28] Prolonged agonist exposure induces nAchRs and $GABA_ARs$ to undergo a slower conformational transition to one or more nonconducting desensitized state(s).[26,29,30] For both receptor families, the processes of agonist binding, channel gating, and desensitization may be most simply represented by the following kinetic scheme:

$$2A + R \longleftrightarrow A_2R \longleftrightarrow A_2O \longrightarrow A_2D$$

agonist binding channel opening desensitization

where A is the agonist, R is the closed resting state, A_2R is the closed preopen state, A_2O is the open channel state, and A_2D is the closed desensitized state(s). As required by this scheme and demonstrated by our data, *Torpedo* nAchR apparent desensitization rates and $GABA_AR$ currents increase with agonist concentration before reaching a plateau. An apparent agonist K_d may then be defined from such agonist concentration–response curves as the agonist concentration required to induce a half-maximal response. In the current study we used two different methods for determining the apparent agonist K_d. For the *Torpedo* nAchR, we used a nonflux fluorescence assay that determines the apparent agonist K_d from the apparent rate of acetylcholine-induced desensitization measured over a wide range of acetylcholine concentrations. This technique (rather than ion flux techniques) was chosen to measure the apparent agonist K_d because many anesthetics not only increase the *Torpedo* nAchR's apparent agonist affinity, but also inhibit ion flux through it.[8,31,32] Such inhibition can mask anesthetic-induced agonist potentiation when electrophysiologic or radiotracer flux techniques are used.[9] For the $GABA_AR$ (which is not as potently blocked by anesthetics), we used a standard electrophysiologic approach and calculated the apparent agonist K_d from the GABA concentration dependence of the measured peak current. We chose cyclopropane and butane as representative nonhalogenated alkane anesthetics because human potency data are available for both of these anesthetics and because concentrations in excess of those required to induce anesthesia may be readily achieved.

We have previously demonstrated that in the *Torpedo* nAchR, isoflurane and normal alcohols increase the apparent rate of desensitization induced by low concentrations of agonist.[33] Subsequent studies using a wide range of agonist concentrations demonstrated that these anesthetics induce a leftward shift in acetylcholine–response curves for desensitization.[8,9] This is indicative of a reduction in the *Torpedo* nAchR's apparent agonist K_d. In these studies, we found that anesthetics reduced the apparent agonist K_d by approximately half at clinical anesthetic concentrations. Furthermore, we observed that the apparent agonist K_d decreased logarithmically with anesthetic concentration without clear evidence of saturation even at relatively high concentrations. Consequently, high concentrations of isoflurane and normal alcohols can reduce the *Torpedo* nAchR's apparent agonist K_d by 10–100-fold. One of the principle results of this study is that even at high concentrations that are more than sufficient to induce anesthesia, cyclopropane and butane fail to potentiate the effects of low agonist concentrations or to reduce the apparent agonist K_d of the *Torpedo* nAchR.

At clinically relevant concentrations, a wide range of general anesthetics also potentiate the actions of low GABA concentrations on $GABA_ARs$.[11] In common with the *Torpedo* nAchR, studies using a range of agonist concentrations indicate that this potentiation reflects a reduction in the $GABA_AR$'s apparent agonist K_d.[3,34] Because GABA is a major inhibitory neurotransmitter in the central nervous system, it is thought that the central nervous system–depressant effects of general anesthetics may result from their abilities to potentiate GABA's actions on $GABA_ARs$. In light of our data demonstrating

that *Torpedo* nAchRs are unaffected by clinically relevant concentrations of cyclopropane and butane, and given the similarity in structure and function between the nAchR and $GABA_AR$, we hypothesized that $GABA_ARs$ might also be insensitive to clinically relevant concentrations of these cyclopropane and butane. To test this hypothesis, we studied the effect of 1.6 MAC concentrations of cyclopropane and butane on the apparent agonist affinity of the $GABA_AR$. Figures 4A and B demonstrate that at these concentrations, both cyclopropane and butane fail to reduce the $GABA_AR$'s apparent agonist K_d. Thus, not only are *Torpedo* nAchRs and $GABA_ARs$ both sensitive to agonist potentiation by isoflurane and normal alcohols, they are both relatively insensitive to agonist potentiation by cyclopropane and butane. This correlation between anesthetic action on the *Torpedo* nAchR and $GABA_AR$ suggests that the underlying molecular mechanism(s) responsible for agonist potentiation in both superfamily members is the same. Furthermore, the inability of cyclopropane and butane to reduce the apparent agonist K_d of the $GABA_AR$ at a concentration that induces anesthesia strongly suggests that other molecular mechanisms likely account for their *in vivo* anesthetic activities. Although we have not yet studied the activities of nonhalogenated anesthetics on other superfamily members (5-hydroxytryptamine$_3$ or glycine receptors), the observation that they do not potentiate agonist actions on either the nAchR or the $GABA_AR$ suggests that they also fail to potentiate these LGICs. As these anesthetics potently inhibit neuronal nAchRs and NMDA receptors, these ion channels must be considered to be potentially relevant targets of nonhalogenated alkane anesthetics.

A recent study by de Sousa *et al.*[35] identified xenon as another anesthetic that does not potentiate the $GABA_AR$ at a clinically relevant concentration (1.1 MAC). In an accompanying editorial, Harrison[36] suggested that xenon may fail to potentiate agonist action because it is unable to participate in hydrogen-bonding interactions with the receptor. Such interactions, which are possible for ether, alcohol, and nearly all halogenated alkane anesthetics, have been suggested to augment binding affinity to a model protein by an order of magnitude.[37] Our data demonstrating that at 1.6 MAC, cyclopropane and butane have no effect on the $GABA_AR$'s apparent K_d is generally consistent with Harrison's suggestion as these two anesthetics are also incapable of forming hydrogen bonds. Alternatively, Cafiso *et al.* and Pohorille *et al.* have suggested that an anesthetic's dipole moment mediates its interactions with membrane targets.[38–41] Because nonhalogenated alkanes (and xenon) are among the very few anesthetics that do not possess a permanent dipole moment, we cannot rule out a critical role for the dipole moment in modulating anesthetic action on the *Torpedo* nAchR and $GABA_AR$.

In summary, our studies show that, at concentrations that are sufficient to induce anesthesia, neither cyclopropane nor butane significantly increases the apparent agonist affinities of two representative members of the superfamily of LGICs, the *Torpedo* nAchR and the $GABA_AR$. Such findings strongly suggest that agonist potentiation of LGICs does not account for the anesthetic activities of nonhalogenated alkane anesthetics.

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